Isolation of Flower-Related Genes From *Aerides japonicum* By Suppression Subtractive Hybridization

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ABSTRACT

The exploration of genes related to flower pigmentation by suppression subtractive hybridization (SSH) were investigated. The 16 newly generated sequences of *Aerides japonicum* by SSH are deposited in the GenBank, DDJB, and EMBL. These genes were all related to pigmentation with one exception of metallothionein-like protein. Especially, glutathione S-transferase played a crucial role in stabilization and detoxification of anthocyanins by transport to the vacuole. The other genes such as dormancy-associated protein, lipid transfer proteins, germin-like protein gene and 3-hydroxy-3-methylglutaryl-CoA synthase were related to biosynthesis of anthocyanins.

INTRODUCTION

Few plants surpass the orchids in distribution throughout the world, variability of growth habits, and magnificent spectrum of colors produced by their flowers and leaves (Arditti and Ernst, 1971; Strauss and Arditti, 1972). To a very large extent, this wealth of colors is due to anthocyanins. Singly or in combinations with other pigments, they contribute to delicate pastels, dazzling yellows, brilliant reds, dull browns, and exciting purples (Arditti and Fisch, 1977).

Anthocyanins are flavonoids derived from 2-phenylbenzopyran (Bate-Smith, 1949). Flavonoids are phenolic compounds that include a wide range of colored substances. The most widespread group of pigmented flavonoids are the anthocyanin, which range in color from yellow to red and magenta (Arditti and Fisch, 1977; Taiz and Zeiger, 1991). Anthocyanins are glycosides that have sugars at position 3 and sometimes elsewhere. Without their sugars, anthocyanins are known as anthocyanins (The non-water-soluble colored part of the molecule, also called the agylecone) (Taiz and Zeiger, 1991; Salisbury and Ross, 1992).

Subtractive cDNA hybridization has been a powerful approach to identify and isolate cDNA of differentially expressed genes. Diatchenko et al.(1996) reported a new PCR-based cDNA subtraction method, termed suppression

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subtractive hybridization (SSH), and demonstrated its effectiveness. They suggested that the SSH technique is applicable to many molecular genetic and positional cloning studies for the identification of disease, developmental, tissue-specific, or other differentially expressed genes.

This work was performed on *Aerides japonicum* in relation to the inheritance of floral pigmentation. The present studies included the exploration of genes related to flower pigmentation by suppression subtractive hybridization (SSH) in *A. japonicum* compared to *A. japonicum* Alba.

MATERIALS AND METHODS

Subtractive hybridization

Two microliters of driver double strand cDNA (600 ng) from *A. japonicum* was added to each of two tubes containing 2 μL of adaptor 1- and 2-ligated tester cDNA (20 ng) from *A. japonicum* Alba. the samples were mixed, ethanol precipitated, and then resuspended in 1.5 μL of hybridization buffer (50mM HEPES, pH 8.4, 0.5M NaCl, 0.02 mM EDTA, pH 8.8, 10% PEG 8000). The cDNA were denatured and then allowed to anneal for 8h at 68℃. After first hybridization, two samples were combined and a fresh portion of heat-denatured driver in 1.5 μL of hybridization buffer was added. The sample was allowed to hybridize for an additional overnight at 68 ℃. The final hybridization was then diluted in 200 μL of dilution buffer (20 mM HEPES, pH8.3, 50 mM NaCl, 0.2 mM EDTA), heated at 72℃ for 7 min and stored at -20 ℃.

PCR amplification and subcloning of the product into TA vector

For each subtraction, two PCR amplification was performed. The primary PCR was conducted in 25μL. It contains 1 μL of diluted subtracted cDNA, 10 μM PCR primer, and 22 μL of PCR master mixture prepared using the Advantage cDNA PCR core Kit (Clonetech Co.). PCR was performed with the following conditions; for 7 min at 75 ℃ and 30 cycles at 94 ℃ for 10 sec, 66 ℃ for 30 sec, 72 ℃ for 90 sec. The diluted PCR product was then used as a template in secondary PCR for 10 cycles under the same conditions used for the primary PCR, except PCR primer 1 was replaced with nested PCR primer PN1 and PN2. The PCR products were analyzed by 1.4% agarose gel electrophoresis.

The subtracted library cDNA was inserted directly into pGEM-T Easy vector using a TA cloning kit (Promega) and then introduced into bacterial host strain *Escherichia coli* JM109.

Analysis of the subtracted cDNA clone

DNA sequencing was performed by automated means at the Bionet Co. Nucleic acid homology searches were performed using the BLAST program through internet servers at the National Centers for Biotechnology Information. Alignment of genes was performed using DNAStar program.

RESULTS AND DISCUSSION

The entire population of hybridized DNA was then subjected to two rounds of PCR to amplify the desired differentially expressed sequences. In the primary PCR, only the molecules with different adaptors at each end can be exponentially amplified (templates with the same adaptor at both ends will form pan-like structures which were suppressed in

PCR). In the secondary PCR, nested primers were used to reduce any background while further enriching the differentially expressed sequences (Fig. 1).

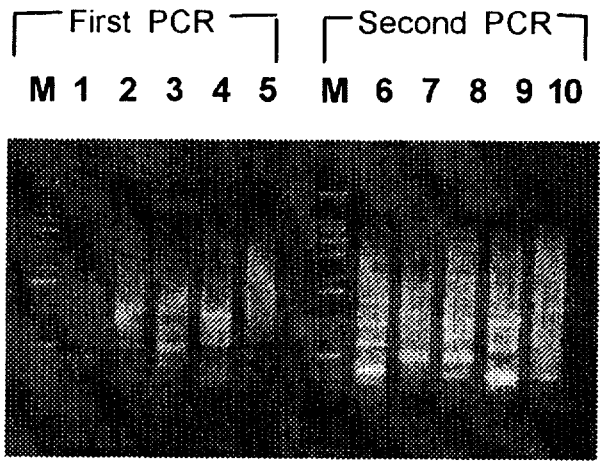

Fig. 1. Amplification products generated first (lane 1 to 5) and second (lane 6 to 10) PCR of unsubtractive (lane 2, 3, 7, 8) and subtractive hybridization (lane 4, 5, 9, 10). M: 1 kb ladder, Lane 1 and 6: PCR control of subtracted skeletal muscle tester, lane 2 and 7: unsubtracted common type cDNA digested with *Rsa*I, lane 3 and 8: unsubtracted alba type cDNA digested with *Rsa*I, lane 4 and 9: subtract hybridization of *A. japonicum*, lane 9 and 10: subtract hybridization of *A. japonicum* alba.

After secondary PCR, the enriched subtracted DNAs were inserted into a TA cloning vector and obtained approximately 300 to 400 white colonies. Differential expression of sequences were appeared various size from 200 to 1500 bp (Fig. 2).

The 16 newly generated sequences of *A. japonicum* are deposited in the GenBank, DDJB, and EMBL under accession numbers listed in Table 1. These genes are all related to pigmentation with one exception of

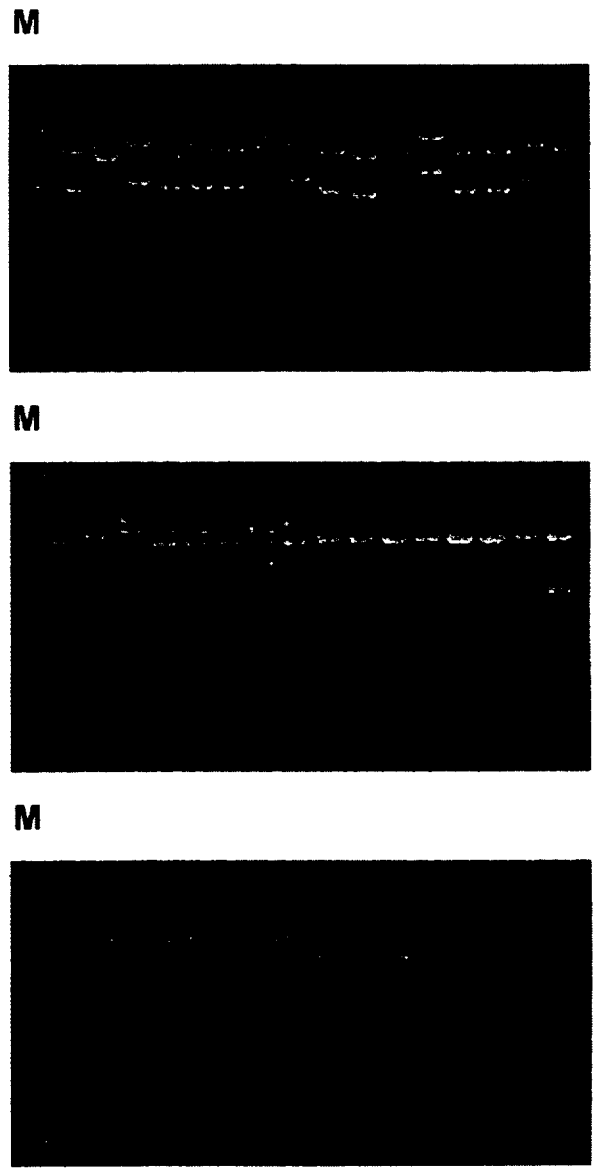

Fig. 2. Restriction enzymes digestion with *Eco*RI of cDNA clones obtained by suppression subtractive hybridization of *A. japonicum*. M: 1 kb ladder

metallothionein-like protein. The occurrence of metallothioneins (MT) or of metallothionein-like metalloproteins (MTL) has been reported for many species of vertebrates and invertebrates (Roesijadi, 1992). These metalloproteins were first discovered and characterized in mammals (Margoshes and Vallee, 1957). They exhibit a low molecular weight, a high cycteine content with a unique distribution of its residues, an

Table 1. *A. japonicum* flower related genes of suppression subtractive hybridization (SSH) generated by BLAST analysis.

No.	Genes of suppression subtractive hybridization	cDNA length of cloning (bp)	ORFs (bp)	Genbank Accession No.
1	Non specific lipid transfer protein precursor	439	32-388	AF198157
2	Lipid transfer protein LPT III	363	31-363	AF198160
3	Phospholipid transfer protein precursor	297	62-297	AF198164
4	Phospholipid transfer protein	657	42-311	AF198162
5	Metallothionein-like protein	360	13-132	AF198158
6	DNA binding protein	307	9-307	AF198159
7	Cytoplasmic aldolase	527		AF198161
8	Profilin mRNA	403		AF198163
9	Dormancy-associated protein	342	79-342	AF198165
10	Flavodoxin gene	455	102-455	AF198166
11	Plasma membrane intrinsic protein	470		AF198167
12	Phospholipid transfer protein mRNA	697	65-427	AF198168
13	3-hydroxy-3-methylglutaryl-CoA-synthase	342	27-342	AF198169
14	Germin-like protein gene	255		AF198170
15	Glutathione S-transferase	164		AF198171
16	Pollen specific mRNA	253		AF198172

absence of aromatic amino acids, heat-stability and a selective capacity to bind metal ions such as copper, zinc, cadmium and mercury by the formation of tetrahedral metal-thiolate complexes organized in two oligonuclear clusters (Otvos and Armitage, 1980) which are biologically unique (Vallee and Maret, 1993).

The anthocyanin biosynthetic pathway is remarkably conserved among flower plants. Anthocyanins share a common biosynthetic origin and core structure, with species-specific decoration of the core by hydroxylation, methylation, sugar addition or acylation. These modifications result in the diverse red, blue and purple colors in the vacuoles of flowers, fruits and leaves. Despite the extraordinary chemical diversity of anthocyanins, these cytoplasmically synthesized molecules are all ultimately localized to the vacuole (Marrs, 1995). It is result from that anthocyanins are toxic substances that could be harmful to the cell. To cope with this potential problem, anthocyanins are stabilized and detoxified by transport to the vacuole. Recent evidence indicates that glutathionation and active transport of the conjugate by a glutathione S-X pump play a critical role in this process (Marrs et al., 1995). They showed that the *Bz2* gene of maize encodes a type III glutahione S-transferase (GST). *Bz2* mutants lacking this activity accumulate anthocyanins in the cytosol, conferring a tan-bronze phenotype. In this step, several kinds of lipid transfer proteins act on a acceptor to DNA binding protein (Mol et al., 1999).

There have been reported that anthocyanins are increased by abscisic acid. ABA appears to act as the dormancy-inducing hormone, as indicated by an observed correlation between levels of endogenous ABA and the physiological state of seed (Taiz and Zeiger, 1991). A role of ABA in dormancy is also indicated by the observation that exogenous ABA prevents germination and induces dormancy. In this study, dormancy-associated protein and germin-like protein gene were found in *A. japonicum*, which indicated that they may be related to anthocyanins. Alleman and Kermicle (1993) reported that R gene regulates the timing and tissue-specificity of anthocyanin deposition during maize development. The patterns of somatic variegation of these mutants, resulting from excision of *Ds*, define a spectrum of phenotypes ranging from sparse to dense variegation. The sparsely variegated mutants produce few germinal revertants but relatively many stable null derivative allele. The densely variegated mutants produce many germinal revertants and few stable null derivatives.

The enzyme 3-hydroxy-3-methylglutaryl-

coenzyme A (HMG-CoA) plays a key role in the synthesis of various sterols and isoprenoids in eukaryotic cells. Sterols are structural components of plasma membranes, wherease terpenoids are involved in various functions including respiration, glycosylation and signal transduction. The isoprenoid pathway of plants is characterized by its diversity, which leads to the synthesis of various molecules such as phytohormones and secondary metabolites. There are two alternative biosynthetic pathways for ABA. In the indirect pathway, ABA is derived from a 40-carbon compound, violaxanthin. In the direct pathway, ABA is derived from a 15-carbon precursor, farnesyl pyrophosphate which are derived from mevalonic acid. In the mevalonate pathway, the conversion of acetyl-CoA to HMG-CoA is mediated by HMG-CoA synthase.

Anthocyanin is synthesized through a dozen reaction steps, where several enzymes including chalcone synthase (CHS), the first enzyme for anthocyanin production, are activated by light, following several-hour-phase. Flavodoxins are a group of small flavoproteins which function as low-potential one-electron carriers and contain a non-covalently bound flavin mononucleotide (FMN) cofactor, which are related to photosystem I and electron transfer.

All biochemical processes that determine form and function of plants are the result of information encoded within the DNA sequence of the genome and the interaction of that information with the environment. This information is converted into biochemical activity and the resulting macromolecular structure of the plant through biosynthesis of specific enzymes or proteins via transcription and translation. In other words, all of the morphology and physiology of plants is based on metabolic processes, and in turn, all of the metabolic processes are the result of the conversion of genetic information into the enzymes and proteins that control metabolism.

In conclusion, these results suggest that the pigment of *A. japonicum* flowers is a cyanidin-based anthocyanins, and expressed by some genes related to anthocyanin biosynthesis such as glutathione S-transferase, dormancy-associated protein, lipid transfer proteins, germin-like protein gene and 3-hydroxy-3-methylglutaryl-CoA synthase. They are inherited through two hybrids dominantly. To obtain *A. japonicum* alba massively, it needs to cross between *A. japonicum* alba tehmselvels and propagate by seeds *in vitro*.

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